

Antimicrobial activity of filamentous fungi isolated from highly antibiotic-contaminated river sediment

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Background: Filamentous fungi are well known for their production of substances with antimicrobial activities, several of which have formed the basis for the development of new clinically important antimicrobial agents. Recently, environments polluted with extraordinarily high levels of antibiotics have been documented, leading to strong selection pressure on local sentinel bacterial communities. In such microbial ecosystems, where multidrug-resistant bacteria are likely to thrive, it is possible that certain fungal antibiotics have become less efficient, thus encouraging alternative strategies for fungi to compete with bacteria.

Methods: In this study, sediment of a highly antibiotic-contaminated Indian river was sampled in order to investigate the presence of cultivable filamentous fungi and their ability to produce substances with antimicrobial activity.

Results: Sixty one strains of filamentous fungi, predominantly various *Aspergillus* spp. were identified. The majority of the *Aspergillus* strains displayed antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, extended-spectrum beta-lactamase-producing *Escherichia coli*, vancomycin-resistant *Enterococcus faecalis* and *Candida albicans*. Bioassay-guided isolation of the secondary metabolites of *A. fumigatus* led to the identification of gliotoxin.

Conclusion: This study demonstrated proof of principle of using bioassay-guided isolation for finding bioactive molecules.

Keywords: *secondary metabolites; Aspergillus; gliotoxin*

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Modern and global healthcare today face the problem of multi-resistant bacteria and the threat of epidemics and pandemics (1–3). Many bacterial species have developed resistance mechanisms against several classes of antibiotics in a relatively short period of time after the clinical introduction of antibiotics. Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) are all examples of bacteria that are difficult to treat due to the lack of effective antibiotics. The pace of development of genuinely new antibiotics

is disappointingly slow, however, hampered not least by the limited payback for research investments in this type of drugs. After the identification and mass production of penicillin in the 1940s with its effect on Staphylococci and Streptococci, a menace often causing life-threatening infection, many efforts have been undertaken to identify and improve new betalactam antibiotics in order to counteract various resistance mechanisms emerging among human pathogenic bacteria (4, 5). However, several resistance mechanisms against vancomycin have been identified (6, 7) and new generations of carbapenems and cephalosporins have subsequently

been developed (6). These four antibiotics are secondary metabolites produced by micro-organisms and were originally found on a contaminated culture plate (8), in soil (9, 10) and in seawater near a wastewater treatment plant (11), respectively.

Exploring environmental microbes and elucidating what antimicrobial substances they produce is indeed a classical strategy to find novel antibiotics. Recently, environments contaminated with extraordinary high levels of broad-spectrum antibiotics have been identified (12–15). Using metagenomic DNA sequencing, Kristiansson et al. 2011 identified 37 antibiotic resistance genes in bacteria from Indian river sediment highly contaminated with broad-spectrum antibiotics, whereas only two resistance genes were found in less contaminated Swedish river sediment. It is possible that fungi, living in environments where multi-resistant bacteria are thriving, have been pushed to rely on mechanisms other than the endogenous production of the known classes of antibiotics. Therefore, our aim was to study the filamentous fungi isolated from highly antibiotic-contaminated sediment and investigate their production of substances with antimicrobial activity against both susceptible and resistant strains of bacteria.

Material and methods

River sediment was sampled in 2008 from the Isakavagu stream downstream from the outlet of the common effluent treatment plant Patancheru Enviro Tech Ltd (PETL). The treatment plant receives about 1500 m³ wastewater daily, mostly from local bulk drug manufacturers, and their effluent is highly contaminated with a broad range of active pharmaceutical ingredients, particularly broad-spectrum fluoroquinolone antibiotics (12, 13, 15). Ciprofloxacin has been detected at concentrations up to 31 mg/L in the effluent (15), and up to 914 µg/g in the river sediment (12). After sampling, the sediment was immediately transferred to 4°C and brought in an unbroken cold chain to the laboratory in Sweden. The specimen was cultured with sterile cotton swabs on Sabouraud dextrose agar (Oxoid), Potatodextrose agar (BD) and malt agar plates. The plates were incubated at 30°C until colonies were visible. The filamentous fungi were re-cultured on Potatodextrose slopes at 30°C to ensure purity, and the isolates were identified by macroscopic and microscopic observation of cultures. The isolates were then stored at –70°C until culture filtrates were produced.

Antibacterial activity of the culture filtrates of filamentous fungi were tested against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and four clinical isolates: vancomycin-resistant *Enterococcus faecalis* van A V4932, carbapenem-resistant *Klebsiella pneumoniae* 91.07, methicillin-resistant *Staphylococcus aureus* 2.08, and ESBL-producing *Escherichia coli* 4.09.

The culture filtrate of *A. fumigatus* no. 1 was also tested against *Pseudomonas aeruginosa* 97.09 (resistant against β-lactam antibiotics, gentamicin, amikacin, ceftazidime, ciprofloxacin) and *Candida albicans* ATCC 90028.

Conidia were taken with a sterile cotton swab from a fresh mature culture (5 days) of the isolate and transferred to 10 mL RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid (Sigma). The conidial suspension was adjusted by enumeration in a haemocytometer chamber to a final concentration of 2–6 × 10⁶ conidia/mL. The cultures were incubated stationary at 30°C and after 5 days the culture medium was sterilized using Acrodisc® syringe filter 0.2 µm (Pall Life Sciences). The filtrates were refrigerated prior to testing.

Overnight cultures of test bacteria were suspended in sterile 0.9% saline and diluted in RPMI 1640 medium to a final concentrations of 10⁴ CFU/mL confirmed by colony counts on blood agar plates (Labfab/Alpha) after incubation at 37°C. Seven wells in 96 round-bottomed sterile plastic microtitration plates (Corning Costar Europe, The Netherlands) were filled with 100 µL of each filtrate. Thereafter the wells were inoculated with 25 µL of each bacterial suspension. Wells containing filtrate and filtrate-free RPMI 1640 served as sterility controls, and wells containing bacterial suspensions in RPMI 1640 medium served as growth controls. After 24 and 48 h incubation of the microtitration plates at 37°C the wells were read visually by recording the degree of growth in each well. The bactericidal effect was tested by plating 10 µL from each well without visible growth onto blood agar plates. Agar plates were incubated at 37°C over night and CFU/mL were counted. Dilutions in RPMI 1640 of culture filtrate of *A. fumigatus* no. 1 were tested as described above. The production and testing of the culture filtrates revealing bactericidal activity were performed twice.

Separation was performed with an Äkta Basic 10 high performance liquid chromatography (HPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden). Reverse-phase chromatography was done with a 250 × 4.6 mm Ace C18 column (Ace, UK) packed with 5 µm particles at a flow of 1 mL/min. The UV-900 Detector operated at 215, 254, and 280 nm. For separation, 2 mL of the filtrate was injected to the RP-HPLC system with a gradient from 5 to 80% acetonitrile over 32 min. Seven fractions were collected, freeze dried, and redissolved in 2 mL RPMI 1640. The bactericidal activity of the fractions was bio-assayed as described above.

LC-MS analyses were made with a Waters CapLC System and a Micromass Q-TOF micro mass spectrometer, using MassLynx 4.0 software (Waters, MA, USA), using a Jupiter C18, 5 µ, 150 × 1.0 mm column from Phenomenex (CA, USA). The following settings were used: desolvation gas flow 300 L/h and at 300°C, cone gas

100 L/h, source temperature 100°C, capillary voltage 3000 V, cone voltage 10 V, mass range 35–800 m/z and scan time 1 sec. Lock spray with Tyr-Gly-Gly-Phe-Leu (556.2771 m/z) was injected at 30 µL/min. For reference tests, gliotoxin produced by *Gliocladium fimbriatum* (Sigma) was used.

NMR spectra were obtained by using a Varian 400 MHz AS400 spectrometer from Oxford Instruments (UK). Samples were dissolved in CDCl₃ to a concentration of 5 mg/mL at 25°C. Tetramethylsilane (TMS) was used as internal standard, and all ¹H chemical shifts are presented as δ values in parts per million (ppm).

Results

From the sediment specimen 61 different fungal isolates were identified (Table 1). The bactericidal effects of the fungal culture filtrates are displayed in Table 2. Of the 31 *Aspergillus* spp. isolates, 20 showed bactericidal activity. Five of six *A. fumigatus* and four of six *A. flavus* isolates had bactericidal effect against all tested bacterial strains. *A. niger* isolates and *Trichoderma* spp. isolates showed bactericidal effect against gram positive bacterial strains. The culture filtrate of *A. fumigatus* no. 1 revealed bactericidal activity against *E. faecalis* VRE van A V4932, MRSA 2.08, *E. coli* ESBL 4.09 and *P. aeruginosa* 97.09 in dilutions down to 1:512, 1:256, 1:16 and 1:1, respectively. The filtrate diluted 1:8 showed antifungal activity against *C. albicans* ATCC 90028. Because of its strong antimicrobial activity the culture filtrate of *A. fumigatus* no. 1 was chosen for further purification and characterization of the active metabolite.

Table 1. Diversity of the 61 filamentous fungi isolated from the antibiotic-contaminated sediment

Species	No. of strains
<i>Alternaria</i> sp.	2
<i>Aspergillus clavatus</i>	1
<i>A. flavus</i>	6
<i>A. fumigatus</i>	6
<i>A. glaucus</i>	1
<i>A. niger</i>	8
<i>A. terreus</i>	4
<i>Aspergillus</i> sp.	5
<i>Fusarium</i> sp.	5
<i>Mucor</i> sp.	5
<i>Paecilomyces crustaceus</i>	2
<i>Penicillium</i> sp.	1
<i>Phoma glomerata</i>	1
<i>Phoma</i> sp.	4
<i>Rhizopus</i> sp.	3
<i>Trichoderma</i> sp.	5
Unidentified zygomycete	2

The chromatogram (Fig. 1) from HPLC separation revealed four main peaks, eluting at 22.2 (compound 1), 22.7, 25.2 (compound 2), and 22.9 (compound 3) min, which were not present in plain RPMI 1640 medium. The first peak had the strongest antimicrobial activity and a higher UV absorption than the three others. Compound 1, 2 and 3 were all freeze dried as a white powder. Their mass spectra were very similar to each other, and all corresponded to the spectrum of gliotoxin.

Gliotoxin (Fig. 2) is a toxin known to be secreted by *A. fumigatus* and a few other fungi, and it has a mass of 326.0395 Da. With the mother ion at 327.0331 m/z (calculated mass for C₁₃H₁₄N₂O₄S₂+H⁺ is 327.0473), the 309.0729, 263.0892, 245.0853 and 227.0798 m/z ions correlates to [M-OH]⁺, [M-2S]⁺, [M-2S-H₂O]⁺, and [M-2S-2(H₂O)]⁺ respectively.

A coinjection of compound 1 and reference gliotoxin was made, revealing no extra peaks. The set of ions from MS spectra was identical, with the exception of the higher amount of ions at 309.07 m/z for compound 1. A sufficient amount of compound 1 was isolated to run ¹H NMR spectroscopy and correlation spectroscopy (COSY). Proton assignment was made on compound 1 (Table 3).

A few differences were observed for protons in position 3a and 6, and are interpreted as a consequence of their proximity to OH groups, and small differences in pH. The complete extraction procedure is described in Fig. 3.

Discussion

We show here that filamentous fungi, thriving in an environment contaminated with extraordinary levels of broad-spectrum antibiotics and accordingly inhibited by antibiotic-resistant bacteria, are able to produce secondary metabolites with bactericidal activity. Most interestingly, some fungi produced substances with activity against several clinically important multi-resistant bacterial strains for which few alternative treatment strategies are available today. It is possible that secondary metabolites, with modes of action differing from those extensively explored for clinically used antibiotics, have been favored in environments polluted from antibiotic manufacturing. The secondary metabolite found here, gliotoxin is produced by *A. fumigatus* and evinces antitumor activity (16). Due to its toxicity towards mammalian cells (17) it has not been considered a candidate as an antimicrobial agent.

Our results suggest that gliotoxin acts as a potent secondary metabolite with antimicrobial activity. The dominant class of antibiotics detected in the water and sediment were quinolones (12, 13, 15), a group of substances that is not naturally produced by filamentous fungi. Although different types of environments have been extensively explored in the past, we are not aware

Table 2. The bactericidal effect of the fungal culture filtrates against susceptible and resistant bacterial strains

Species	Isolate no.	<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>E. faecalis</i> V4932	<i>K. pneumoniae</i> 91.07	MRSA 2.08	<i>E. coli</i> ESBL 4.09
<i>A. fumigatus</i>	1, 6, 13, 44, 50	+	+	+	+	+	+
<i>A. fumigatus</i>	46	+	+	+	+	+	no
<i>Aspergillus</i> sp.	15	+	no	+	+	+	no
<i>A. flavus</i>	2, 5, 21, 58	+	+	+	+	+	+
<i>A. flavus</i>	4	+	+	+	+	+	no
<i>A. flavus</i>	61	+	no	+	no	+	no
<i>A. niger</i>	28, 30–32, 34, 37, 56	+	no	+	no	+	no
<i>Trichoderma</i> sp.	12, 60	+	no	+	no	+	no

+ indicates $\geq 99.98\%$ bactericidal effect of the filtrate compared to growth control; no = no bactericidal activity

of any other initiative trying to identify novel antibiotics in heavily antibiotic-contaminated environments. Indeed, most of the *A. fumigatus*, *A. flavus* and *Aspergillus* spp. strains from this polluted environment produced secondary metabolites with broad and strong bactericidal effects. Our bioassay-guided method made it possible to test the antimicrobial activity of the isolated fungi against several microorganisms simultaneously with a relatively small workload. The semi-quantitative analysis

by titration of the filtrate indicated the strength of the antimicrobial activity as well.

The exploration of extreme microenvironments is a proven concept that has become more and more interesting with the newer and better-developed microbiological isolation and identification techniques. Due to the large number of variables in this method, the result may vary. For our settings, some metabolites that would be interesting to study might not be produced at all, or

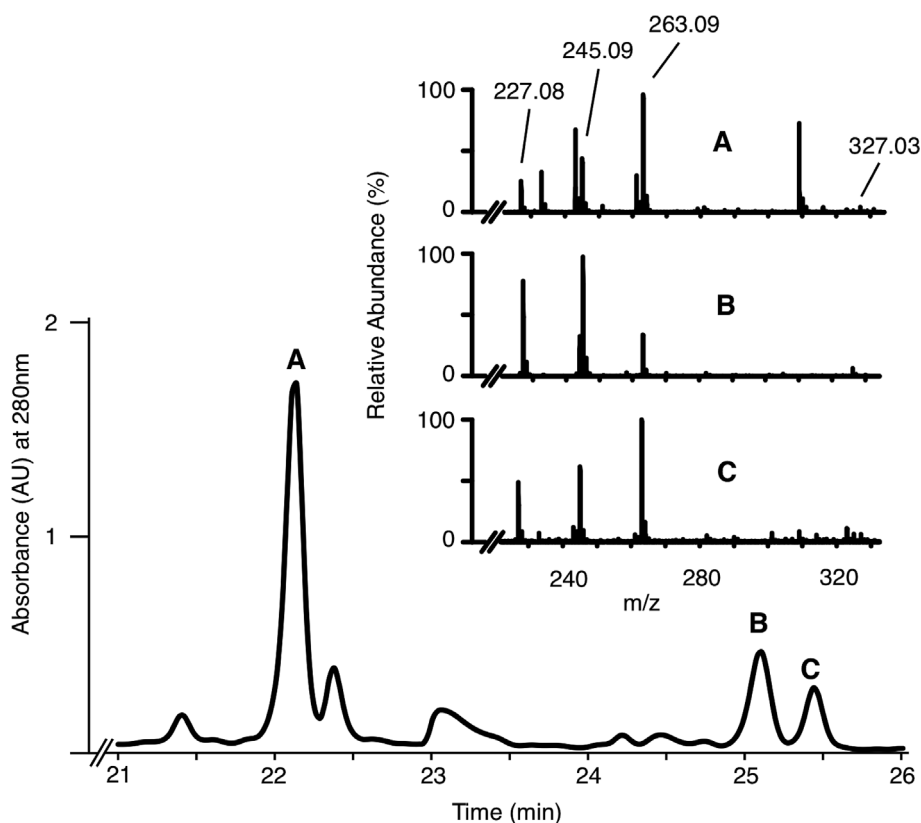


Fig. 1. The analysis of *A. fumigatus* filtrate. Chromatogram recorded at 280 nm and selected mass spectra of filtrate from *A. fumigatus*. From top to bottom, the three spectra origins from the peaks at 22.2, 25.2, and 25.9 min, respectively.

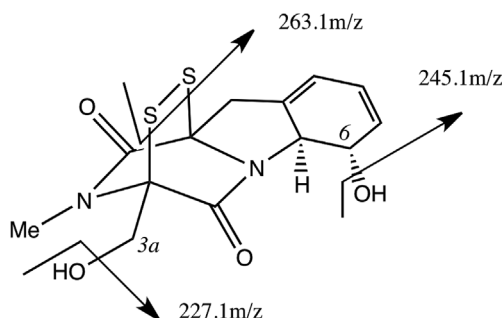


Fig. 2. Compound 1, gliotoxin. The active substance gliotoxin with two numbered carbons and presumed cleavage patterns.

only at very low concentrations. However, the identification of gliotoxin confirms the value of this method. The genus *Aspergillus* consists of almost 200 species which are primarily found in decaying vegetation and soil. Some *Aspergillus* spp. are utilized, e.g. in industrial production of various enzymes (18).

There were most likely more species of filamentous fungi present in the river sediment that were not detected by our culture method. This fact has been highlighted in several other studies (19–21). For example, incubation of the agar plates for isolation of fungi was carried out at 30°C, based on a reasonable ambient temperature in sediment of the Isakavagu stream at the time of sampling. However, this approach may select for more thermophilic fungal species and could explain that the majority of the isolated species in this study had their optimal growth temperature at 37–45°C.

A way to partly overcome the difficulties in cultivating fungi from complex environmental samples and search for secondary metabolites is to apply cultivation-independent

Table 3. NMR data for compound 1 and reference gliotoxin dissolved in CDCl₃

Position	Compound 1 ^a		Reference ^a	
	δ _H (J in Hz)	COSY	δ _H (J in Hz)	COSY
2a	3.19, s		3.13, s	
3a	4.81, s		4.75, s	
3a-OH	3.15, s		3.64, br s	
5a	4.41, d (13)	6	4.35, d (12)	6
6	4.27, d (13)	5a	4.18, dd (12, 7.6)	5a
6-OH	5.59, s		5.63, s	
7	5.78, d (8.8)	8	5.71, d (8.4)	8
8	5.93, dd (8.8, 4.8)	7, 9	5.86, dd (8.4, 4.8)	7, 9
9	5.99, m	8	5.92, m	8, 10A
10A	3.75, dm (18)	10B	3.68, dm (18)	9, 10B
10B	2.95, d (18)	10A	2.88, d (18)	10A

^aRecorded at 400 MHz, referenced to residual solvent at δ_H 7.26 ppm.

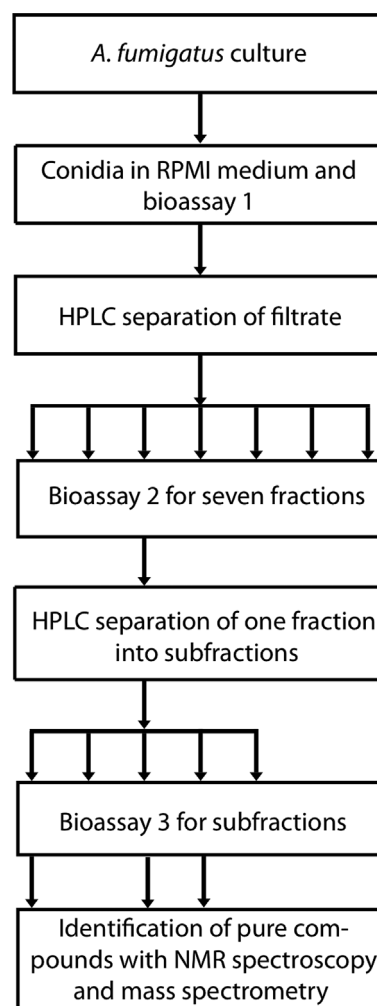


Fig. 3. The isolation process.

approaches. Next-generation DNA sequencing approaches of community DNA is such a method that has opened up new possibilities. As read-lengths and (affordable) sequencing depth is rapidly improving, bioinformatic searches for specific functional domains within the DNA (or RNA) of antibiotic-contaminated microbial communities may very well be a parallel way forward in the search for novel antibiotics.

In the process of the development of new antibiotics, a very large number of small molecules have been screened in laboratories. This study raises the possibility of exploring, not only natural, extreme environments for novel bioactive molecules, as has been extensively done in the past, but also environments that have become extreme in one way or the other due to human activities. Furthermore, we demonstrate proof of principle of using bioassay-guided isolation for finding these molecules. The antibiotic-contaminated sediment of the Isakavagu stream is clearly not the only place where such studies can be carried out.

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